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WHAT IS THE FATE OF XYLEM-TRANSPORTED CO_2 IN KRANZ-TYPE C_4 PLANTS?

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SUMMARY

- High concentrations of dissolved inorganic carbon in stems of herbaceous and woody C_3 plants exit leaves in the dark. In the light, C_3 species use a small portion of xylem-transported CO_2 for leaf photosynthesis. However, it is unknown if xylem-transported CO_2 will exit leaves in the dark or be used for photosynthesis in the light in Kranz-type C_4 plants.

- Cut leaves of *Amaranthus hypochondriacus* were placed in one of three solutions of [$NaH^{13}CO_3$] dissolved in KCl water to measure the efflux of xylem-transported CO_2 exiting the leaf in the dark or rates of assimilation of xylem-transported CO_2^* in the light, in real-time, using a tunable diode laser absorption spectroscope.

- In the dark, the efflux of xylem-transported CO_2 increased with increasing rates of transpiration and [$^{13}CO_2^*$]; however, rates of $^{13}C_{efflux}$ in *A. hypochondriacus* were lower compared to C_3 species. In the light, *A. hypochondriacus* fixed nearly 75% of the xylem-transported CO_2 supplied to the leaf.

- Kranz anatomy and biochemistry likely influence the efflux of xylem-transported CO_2 out of cut leaves of *A. hypochondriacus* in the dark, as well as the use of xylem-transported CO_2^* for photosynthesis in the light. Thus increasing the carbon use efficiency of Kranz-type C_4 species over C_3 species.

Keywords: *Amaranthus hypochondriacus*, CO_2 efflux, C_4 photosynthesis, internally transported CO_2, Kranz anatomy, tunable diode laser absorption spectroscopy, xylem-transported CO_2

INTRODUCTION

In Kranz-type C_4 species, vascular bundles in the leaves are surrounded by bundle-sheath cells, which in turn are surrounded by mesophyll cells (Sage, 2004). Kranz anatomy allows C_4 species to compartmentalize and concentrate CO_2 around ribulose-1,5-

bisphosphate- carboxylase/oxygenase (Rubisco), in the bundle-sheath cells, while isolating phosphoenolpyruvate carboxylase (PEPC), to the mesophyll cells (Hatch, 1987), thus reducing rates of photorespiration by Rubisco and increasing rates of photosynthesis (Hatch, 1987; von Caemmerer & Furbank, 1999). However, there is an energetic cost of regenerating phosphoenolpyruvate (PEP) (Hatch, 1987; Kanai & Edwards, 1999; Ubierna *et al*., 2011), which is required to shuttle CO_2 from the mesophyll cells into the bundle-sheath cells as C_4 acids (Hatch *et al*., 1967). In order for Kranz-type C_4 plants to reduce the energetic cost of regenerating PEP, they must have high bundle-sheath resistance to CO_2 diffusion, or low leakiness (ϕ). Leakiness is the proportion of carbon fixed by PEPC that subsequently diffuses out of the bundle-sheath cells instead of being fixed by Rubisco (Farquhar, 1983; Hatch *et al*., 1995). In Kranz-type C_4 species, CO_2 diffuses through the stomata and then into mesophyll cells where it is equilibrated with HCO_3^- by carbonic anhydrase (CA). There it is assimilated by PEPC and transported as C_4 acids into the bundle-sheath cells for decarboxylation and utilization by Rubisco (Hatch *et al*., 1967). However, studies in C_3 species show that inorganic carbon can also be transported through the xylem and used for photosynthesis by stems (Teskey *et al*., 2008; Bloemen *et al*., 2013a), branches (McGuire *et al*., 2009; Bloemen *et al*., 2013a, b), and leaves (Stringer & Kimmerer, 1993; McGuire *et al*., 2009; Bloemen *et al*., 2013a, b; Bloemen *et al*., 2015; Stutz & Hanson, 2019).

Concentrations of dissolved inorganic carbon ($[CO_2^*]$, the sum of $[CO_2]_{aq}$, $[H_2CO_3]$, $[HCO_3^-]$ and $[CO_3^{2-}]$) in the stems of C_3 trees can be between ~0.05 mmol l^{-1} and ~13 mmol l^{-1} (Teskey *et al*., 2008). This inorganic carbon is generated from cellular respiration and dissolves in the xylem and moves in bulk flow along with water through the plant to the leaf where it is either used for photosynthesis (Stringer & Kimmerer, 1993; Teskey & McGuire, 2002; Teskey *et al*., 2008; Bloemen *et al*., 2013a, b; Bloemen *et al*., 2015; Stutz & Hanson, 2019) or exits via transpiration (Stringer & Kimmerer, 1993; Bloemen *et al*., 2013a; Stutz *et al*., 2017). However, it is not just trees that have high $[CO_2]$ in stems, Stutz *et al*. (2017) found $[CO_2]$ in bolted stems of the herbaceous C_3, *Brassica napus* were 0.7 mmol l^{-1}, showing that xylem-transported CO_2^* (the sum of $[CO_2]_{aq}$, $[H_2CO_3]$, $[HCO_3^-]$ and $[CO_3^{2-}]$) is also concentrated in stems of herbaceous C_3 plants.

In *Populus deltoides* the efflux of xylem-transported CO_2 out of branches and stems of labeled saplings ranges from 82.6% in a low-label treatment (1.4 mmol l^{-1} [$^{13}CO_2^*$]) to 94.4% in a high-label treatment (12 mmol l^{-1} [$^{13}CO_2^*$]) (Bloemen *et al*., 2013a). They demonstrated that most of the uptake was due to photosynthesis in the stem, but that at higher concentrations of xylem-transported CO_2^*, a higher percentage of xylem-transported CO_2 exited the plant, indicating that photosynthesis was unable to keep up with supply and the stem and branch porosity to CO_2 was high enough that the remaining xylem-transported CO_2^* exited the plant before being used.

In cut, *P. deltoides* leaves in the dark ~80% of labeled xylem-transported CO_2 exits the leaf as CO_2 (Stringer & Kimmerer, 1993; Stutz *et al*., 2017). However, the amount of xylem-transported CO_2 that exited cut leaves of the herbaceous C_3 plant *B. napus* in the dark were only between 50% and 75% across the range of [$^{13}CO_2^*$] measured (Stutz *et al*., 2017). This shows that the consumption of xylem-transported CO_2^* in the dark varies between C_3 species and was about two times higher in *B. napus* compared to *P. deltoides* (Stutz *et al*., 2017).

In C_3 plants, utilization of xylem-transported CO_2^* was mostly low compared to the amount of xylem-transported CO_2^* available for photosynthesis in the light. Prior work found that over the course of 48 hours, in *P. deltoides* saplings, only 17.4% and 5.6% of a low- and high-label $^{13}CO_2$ were assimilated by plant tissues; the highest concentrations of the label were found in the branch and stem tissues while only a small portion was found in the leaf tissues from both treatments (Bloemen *et al*., 2013a). Spatial variation in rates of xylem-transported CO_2 assimilation on the scale of an individual leaf demonstrated that most of the label was found in the petiole and the tissues most proximal to the label with a small portion found in minor veins and mesophyll cells (Stringer & Kimmerer, 1993; Bloemen *et al*., 2015). Temporal variation in rates of xylem-transported CO_2^* assimilation demonstrated that when intercellular [CO_2] was low (*i.e.* high irradiance or low [CO_2]), rates of assimilation using xylem-transported CO_2^* were highest. However, when the total rate of assimilation was lowest (*i.e.* low irradiance or low [CO_2]), assimilation of xylem-transported CO_2^* reached the highest proportion of total assimilation (Stutz & Hanson, 2019). While previous studies have focused on how trees and C_3 herbaceous plants utilize xylem-transported CO_2^* for photosynthesis, to our knowledge, no study has focused on the efflux

of xylem-transported CO_2 from C_4 plants in the dark or how C_4 plants use xylem-transported CO_2^* for photosynthesis.

Kranz anatomy may impact the efflux of xylem-transported CO_2 in the dark or influence the use of xylem-transported CO_2^* in the light as a result of one or several possibilities: 1) higher concentrations of PEPC may lead to higher consumption of xylem-transported CO_2^*, when in equilibrium with bicarbonate, in the light, and higher consumption in the dark as it takes 90 to 120 minutes for PEPC to deactivate in the dark in C_4 species compared to C_3 species (Rajagopalan *et al*., 1993), 2) Kranz anatomy may hinder the amount of xylem-transported CO_2 exiting the leaf in the dark compared to C_3 species, and 3) Kranz anatomy may allow C_4 plants to capture more xylem-transported CO_2^* for photosynthesis in the light due to photosynthetic cells tightly surrounding the vascular-bundles. Therefore, the objectives of this study were to estimate: 1) the rate and total efflux of xylem-transported CO_2 exiting a cut leaf of the Kranz-type C_4 species, *Amaranthus hypochondriacus*, in the dark and 2) the rate and contribution of xylem-transported CO_2^* to total assimilation in the light for *A. hypochondriacus*.

These goals were accomplished using a LI-6400 with a custom leaf chamber coupled to a tunable diode laser absorption spectroscope (TDL) to measure how much xylem-transported CO_2 entered a cut leaf and exited the leaf in the dark or was used for photosynthesis in the light using light- and CO_2-response curves (Stutz *et al*., 2017; Stutz & Hanson, 2019). We hypothesized that Kranz anatomy found in *A. hypochondriacus* would prohibit much of the xylem-transported CO_2 from exiting cut leaves in the dark. In the light, we hypothesized that Kranz anatomy would prohibit much of the xylem-transported CO_2 from exiting from the leaf in the light thus leading to higher rates of assimilation of xylem-transported CO_2^* and reduced rates of $^{13}CO_2$ efflux in the light in *A. hypochondriacus* compared to *B. napus*.

MATERIALS & METHODS

Plant propagation and growth

Amaranthus hypochondriacus (L.) was grown under SPYDR 1200 GROW-MAX LED lights (Fluence Bioengineering, Austin, TX, USA) with 500 μmol quanta m^{-2} s^{-1} and a day/night cycle of 12 hours, in a greenhouse at the University of New Mexico in Albuquerque, NM, USA in ambient CO_2. The greenhouse temperature varied between 20ºC-23.9ºC across a day. *A. hypochondriacus* was started from seed. Seeds were sowed in 500 mL pots with Metro-Mix 300 potting soil (Sun Gro Horticulture, Seba Beach, AB, Canada); approximately 30 days after germination, seedlings were transferred to 3.7 L pots filled with Metro-Mix 300 potting soil. Plants were fertilized twice weekly with Peter 20-20-20 fertilizer (Scotts Miracle-Gro, Marysville, OH, USA) and once weekly with chelated liquid iron (ferti-lome, Bonham, TX, USA). Plants were measured between 50 and 80 days after geminating. *Brassica napus* (L. stellar DH GT060615) was propagated and grown according to Stutz *et al*. (2017).

Dark efflux measurements

A LI-6400 (LI-COR Biosciences, Lincoln, NE, USA) was coupled to a tunable diode laser absorption spectroscope (TDL—model TGA 100; Campbell Scientific, Inc., Logan, UT, USA) to measure online $^{12}CO_2$ and $^{13}CO_2$ exchange along with online carbon isotope discrimination. Isotope calibration consisted of a high and low CO_2 tank that spanned the expected range of [CO_2] of each isotopologue for the LI-COR reference and sample (Barbour *et al.,* 2007; Stutz *et al*., 2017) followed by the LICOR reference and sample. Since the TDL measures [$^{12}CO_2$] and [$^{13}CO_2$], the net fluxes of each were calculated as in normal gas exchange. The average precision of the TDL was 0.2 μmol mol^{-1} ± 0.1 SD μmol mol^{-1} for the ^{12}C isotopologue, 0.004 μmol mol^{-1} ± 0.002 SD for the ^{13}C isotopologue, and 0.1‰ ± 0.05 SD for $\delta^{13}C$.

The highest fully expanded leaf was placed in a large (80 cm^2) clear toped, custom leaf chamber with an RGB LED light source (LI-6400-18; LI-COR Biosciences) attached to a LI-6400 at 25ºC (leaf temperature) and 380 μmol mol^{-1} CO_2 reference with the RGB light source set at 1600 μmol quanta m^{-2} s^{-1}. The leaves were photographed, and the projected

leaf area was estimated using ImageJ (US National Institutes of Health, Bethesda, MD, USA). The leaves fit width-wise in the chamber but extended ~4-7 cm beyond the edge of the chamber. The petiole length was between 5 and 10 cm.

Once photosynthesis reached a steady state, the leaf was cut under water and the petiole was placed in a 40 mmol l^{-1} KCl solution. Stomatal conductance and photosynthesis decreased when the leaf was cut but the leaf was kept in the KCl solution until photosynthesis recovered. The KCl solution was swapped for 99% ^{13}C sodium bicarbonate dissolved in 40 mmol l^{-1} KCl at one of three [$^{13}CO_2^*$]: 1.19 (low-carbon—LC), 5.95 (medium-carbon—MC), or 11.9 mmol l^{-1} (high-carbon—HC). Individual leaves were only provided a single [$^{13}CO_2^*$] (Stutz *et al*., 2017). These concentrations span the range of observed values for tree xylem inorganic carbon concentration ($CO_2 + HCO_3^-$). The pH of the KCl/water solution was not controlled and the pH of the $^{13}CO_2^*$ solutions are recorded in Tables S1 and S2. Once the $NaH^{13}CO_3$ is dissolved in water, the inorganic carbon is represented by several species $[CO_2]_{aq}$, $[H_2CO_3]$, $[HCO_3^-]$ and $[CO_3^{2-}]$. Only the sum of the species ($[CO_2^*]$) provided to the leaf is known since the xylem-sap pH and the activities and locations of enzymes such as carbonic anhydrase (CA) are unknown along the pathways through the leaf. However, similar experiments in C_3 species demonstrated that even when very high concentrations were used, over 90% of the supplied ^{13}C could exit the leaf as $^{13}CO_2$ (Stutz *et al*. 2017). Therefore, we assume there is sufficient carbonic anhydrase throughout the leaf to keep inorganic carbon species near equilibrium, and that in the cases where less $^{13}CO_2$ exits the leaf it is likely the inorganic carbon was captured by a carboxylase. In the HC and MC treatments approximately 8 minutes after adding the $^{13}CO_2^*$ a small increase in $^{13}CO_2$ efflux was observed with the TDL and the light on the LI-6400 was turned off. There was no increase in $^{13}CO_2$ efflux under the LC treatment so the light was turned off after approximately 10-15 minutes. Throughout the measurement period, the rate of transpiration was manipulated by switching the LI-COR desiccant between full scrub (*i.c.*, high VPD and higher rates of transpiration) and full bypass (*i.c.*, low VPD and lower rates of transpiration). To further increase the relative humidity when the desiccant was on full bypass, a condensing tube in a water bath (VWR Scientific Products, West Chester, PA, USA) was attached to the LI-COR inlet to decrease VPD to 0.5 kPa or less. Each treatment consists of five replicates.

Estimating the consumption rate of xylem-transported CO_2 in the dark

The background rate of $^{13}CO_2$ respiration ($^{13}C_{RL}$) was calculated by multiplying the rate of ^{12}C leaf respiration ($^{12}C_{RL}$) by 1.1%, the natural abundance of $^{13}CO_2$ in the atmosphere and the tank air fed to the leaf (Griffis *et al*., 2004; Stutz *et al*., 2017). No adjustment was made for photosynthetic discrimination by C_4 plants since it is small and since the addition of labeled ^{13}C in our experiment caused our data to be far from natural abundance. We calculated an expected ^{13}C efflux ($^{13}C_{cal\ efflux}$) that would occur if all the [$^{13}CO_2{}^*$] added to the cut leaf exited the leaf as $^{13}CO_2$. This was accomplished by multiplying the [$^{13}CO_2{}^*$] in the solution fed to the leaves by the rate of transpiration and adding it to the background $^{13}C_{RL}$. We used the ratio of observed $^{13}CO_2$ efflux ($^{13}C_{efflux}$) to $^{13}C_{cal\ efflux}$ to determine the fraction of supplied $^{13}CO_2$ exiting the leaf and expressed it as a percentage. We estimated the rate of consumption of xylem-transported CO_2 by taking the difference between the $^{13}C_{cal\ efflux}$ and observed $^{13}C_{efflux}$. For these calculations, we assumed the transpiration rate along with the rate of ^{12}C and ^{13}C effluxes were constant across one TDL/LI-COR cycle *i.c.* 4 minutes. We excluded the first 45 minutes after the light was turned off for these calculations and monitored transpiration until enough water had been transpired for us to assume that all leaf water had been replaced with the $^{13}CO_2{}^*$ solution that we supplied to the petiole. Data from *B. napus* were modified from Stutz *et al*. (2017).

Light-response curves

The highest fully expanded leaf was placed in a clear topped, 38.5 cm^2, circular custom leaf chamber made to fit an RGB LED light source (LI-COR Biosciences, Lincoln, NE, USA) attached to a LI-6400 at 23ºC (leaf temperature), 380 μmol mol^{-1} CO_2 reference at 1600 μmol quanta $m^{-2}s^{-1}$. Leaves were left in the chamber for approximately 60 minutes before being cut and placed in a 40 mmol l^{-1} KCl solution. As with the dark efflux measurements, the leaf remained in the KCl solution until the rate of photosynthesis recovered and then the KCl solution was swapped for the LC, MC, HC [$^{13}CO_2{}^*$], or control KCl solution. An individual leaf was only provided a single [$^{13}CO_2{}^*$]. The light response curves were measured in the following order: 1600, 1300, 1000, 800, 500, 250, 200, 175, 150, 125, 100, 75, 50, 35, 0 μmol quanta m^{-2} s^{-1}. Five measurements were made at each irradiance

except for the highest irradiance where we waited for the $^{13}CO_2$ efflux to level out and averaged the last five measurements. Five replicate leaves were measured for each [$^{13}CO_2{}^*$] and KCl solutions. The measurements for *B. napus* are described in Stutz & Hanson (2019).

CO_2-response curves

For the CO_2-response curves, the highest fully expanded leaf was placed in the same LI-6400 custom leaf chamber as was used for the light-response curves at 23ºC (leaf temperature) at 1600 μmol quanta $m^{-2}s^{-1}$. The CO_2 reference on the LI-6400 was set with the [CO_2] at the leaf level approximately 400 μmol mol^{-1}, which was between 500 and 650 μmol mol^{-1} CO_2 reference [CO_2] (starting [CO_2]). Leaf-cutting, KCl treatments, and [$^{13}CO_2{}^*$] additions were the same as for the light responses. The leaf was left at the starting [CO_2] until the $\delta^{13}C$ and [$^{13}CO_2$] reading on the TDL stabilized approximately 20 to 30 minutes. The [CO_2] on the LI-6400 reference were applied to the leaf in the following order: starting [CO_2], 200, 100, 50, 150, 300, starting [CO_2], 1000, 2000, 1500, 700 and starting [CO_2]. The light was turned off and leaf respiration was monitored in the dark for at least 30 minutes before using the data, to avoid light enhanced dark respiration (LEDR). Fives leaves from each species were measured for each [$^{13}CO_2{}^*$] and the KCl solution. The measurements for *B. napus* are described in Stutz & Hanson (2019).

Estimating the amount of xylem-transported CO_2 entering the cut leaves and rate of consumption of xylem-transported CO_2 in the light

The rate of xylem-transported CO_2 consumption in the light in the Kranz-type C_4 species, *A. hypochondriacus* was calculated as:

$$Consumption\ in\ light = Rate\ of\ {}^{13}CO_2\ entering\ leaf - {}^{13}C_{pred\ light\ efflux} \quad \text{(Equation 1)}$$

where, the rate of $^{13}CO_2$ entering the leaf is estimated based on the rate of transpiration and the [$^{13}CO_2{}^*$] added to the cut leaf and where $^{13}C_{\text{pred light efflux}}$ is the predicted rate of xylem transported CO_2 exiting the leaf in the absence of assimilation and is estimated from linear regression models of xylem CO_2 efflux in the dark (Stutz *et al*., 2017).

Statistical analyses

We used R (version 3.4.2, R Development Core Team 2017) for all statistical analyses. Analysis of covariance (ANCOVA) was used to compare slopes among [$^{13}CO_2^*$] treatments in *A. hypochondriacus*, followed by Tukey post-hoc multiple comparisons of means to determine which slopes were significantly different among the three treatments. The percentage of xylem-transported CO_2 exiting the leaf and the rate of consumption of xylem-transported CO_2 were compared between species, *B. napus* and *A. hypochondriacus* and among treatments (LC, MC, and HC) using a two-way analysis of variance (ANOVA) followed by Tukey HSD post-hoc comparisons.

For the light- and CO_2-response curves, we used the lme4 R package (Bates *et al*., 2012) to perform linear mixed effects analyses of the relationship between our physiological response variables ($^{12}A_{obs}$, $^{13}A_x$, photosynthetic discrimination ($\Delta^{13}C$), and consumption of $^{13}CO_2^*$ in the light) and [$^{13}CO_2^*$]. We set [$^{13}CO_2^*$] and irradiance or [CO_2] in the light- and CO_2-response curves, respectively, as fixed effects. We structured the model to allow for random intercepts for individual leaves. The amount of $^{13}CO_2^*$ (the sum of $[CO_2]_{aq}$, $[H_2CO_3]$, $[HCO_3^-]$ and $[CO_3^{2-}]$) entering the leaf and consumption in the light were compared to the C_3 species, *B. napus*. For these models [$^{13}CO_2^*$] and species were the fixed effects. We structured the model to allow for random intercepts for individual leaves and irradiance, or [CO_2] in the light- and CO_2-response, respectively. Results were deemed significant at $P<0.05$. No data were transformed based on the distribution of the residuals.

RESULTS

Retention and magnitude of xylem-transported CO_2 in the dark

In the Kranz-type C_4 species, *Amaranthus hypochondriacus*, rates of xylem-transported CO_2 efflux in the dark ($^{13}C_{efflux}$) increased with increasing rates of transpiration and [$^{13}CO_2^*$] (Fig. 1b). The low-carbon (LC) and high-carbon (HC) treatments were significantly different (P<0.001); however, there were no significant differences between the medium-carbon (MC) and HC treatments (P=0.34), or between the LC and MC treatments (P=0.11). The rate of leaf respiration ($^{12}C_{RL}$) increased slightly with increasing rates of

transpiration (Fig. 1a) but was not significantly different across the range of transpiration (P=0.05). Rates of $^{13}C_{efflux}$ were lower than the herbaceous C_3 species, *Brassica napus* whereas rates of transpiration were similar between the two species, (Fig. 1c), in the HC treatment. When transpiration was 0.75 mmol H_2O m^{-2} s^{-1}, $^{12}C_{RL}$ was ~1.5 μmol CO_2 m^{-2} s^{-1} and was not significantly different among [$^{13}CO_2^*$] (P=0.09) (Fig. 2b). However, the rate of $^{13}C_{efflux}$ increased from ~0.018 μmol CO_2 m^{-2} s^{-1} in the LC treatment to ~0.04 μmol CO_2 m^{-2} s^{-1} in the MC and HC treatments (Fig. 2a); there were no significant differences among rates of $^{13}C_{efflux}$ across [$^{13}CO_2^*$] (P=0.06).

How much xylem-transported CO_2 exited in the leaf in the dark?

The amount of $^{13}CO_2^*$ (the sum of $[CO_2]_{aq}$, $[H_2CO_3]$, $[HCO_3^-]$ and $[CO_3^{2-}]$) that entered the darkened leaf and subsequently exited the leaf as $^{13}CO_2$ in the dark was calculated 90 minutes after turning off the light for both *B. napus* and *A. hypochondriacus*. The percentage of xylem-transported CO_2 exiting the leaf in the dark was significantly different between species (P<0.001) and among [$^{13}CO_2^*$] (P<0.001). There were no significant differences in the percentage of xylem-transported CO_2 exiting the leaf in *A. hypochondriacus* or *B. napus* in the LC (P=0.93) or MC (P=0.67) treatments; however, the percentage of xylem-transported CO_2 exiting the leaf was significantly different in the HC treatment (P<0.001) (Fig. 3a, b). In *A. hypochondriacus*, there were no significant differences in the percentage of xylem-transported CO_2 exiting the leaf in the LC (8%), MC (22%), and HC (25%) treatments (P=0.1) (Fig. 3a). In *B. napus* there was a significant difference in xylem-transported CO_2 exiting the leaf in the LC (15%) and HC (74%) (P<0.001) treatments, along with the MC (33%) and HC treatments (P<0.001); however, there was no significant difference between the LC and MC treatments (P=0.1) (Fig. 3b). The rate of consumption of $^{13}CO_2^*$ was significantly different among all [$^{13}CO_2^*$] in *A. hypochondriacus* (P<0.01) (Fig. 3c) and ~5 times greater than *B. napus* across all [$^{13}CO_2^*$] (Fig. 3c, d); however, we did not measure what compounds contain labeled ^{13}C at the completion of the experiment.

Did xylem-transported CO_2 reach the leaf in the light?

In the KCl and LC treatments, photosynthetic discrimination ($\Delta^{13}C$) increased from ~4‰ under high irradiance to ~8‰ and ~5‰ under low irradiance (Fig. 4a). Before placing the cut leaf in the MC or HC treatments $\Delta^{13}C_{obs}$ was ~4‰ (data not shown) but after placing the leaf in the [$^{13}CO_2^*$] solution $\Delta^{13}C_{obs}$ was ~49‰ and ~141‰ (Fig. 4c) in the MC and HC treatments, respectively. Based on $\Delta^{13}C_{obs}$ in the MC and HC treatments, $^{13}CO_2^*$ clearly exited the leaf as $^{13}CO_2$; $\Delta^{13}C_{obs}$ was significantly different among [$^{13}CO_2^*$] (P<0.001) and across irradiance (P<0.001). However, there was no significant difference between the KCl and LC treatments across any [CO_2] the MC and HC treatments were significantly different from the KCl and LC treatments under non-saturating irradiance (P<0.05) (Fig. 4a, c).

In the CO_2-response curves, $\Delta^{13}C$ in the KCl and LC treatments were ~4‰ under high [CO_2]; and increased to ~10‰ under the lowest [CO_2] (Fig. 4b). There were no significant differences between the LC and KCl treatments across any intercellular [CO_2] in the CO_2-response curves. As in the light-response curves, photosynthetic discrimination in the MC and HC treatments was ~4‰ before cutting the leaf (data not shown) but increased to ~60‰ in the MC and ~175‰ in the HC treatment (Fig. 4d). The HC treatment was significantly different from the KCl, LC and MC treatments (P<0.001) when intercellular [CO_2] was less than 200 μmol mol^{-1}.

How much xylem-transported $^{13}CO_2$ entered the leaf?

Using the rate of transpiration, and the concentration of $^{13}CO_2^*$ added to a cut leaf we were able to calculate how much $^{13}CO_2^*$ entered the leaf in both the herbaceous C_3 species, *B. napus*, and the C_4 *A. hypochondriacus*. Rates of $^{13}CO_2^*$ entering the leaf increased with increasing transpiration and increasing [$^{13}CO_2^*$] for both species (Fig. 5). In the light-response curves the highest rates of $^{13}CO_2^*$ entering the leaf occurred when irradiance and stomatal conductance were high, this also coincided with higher rates of carbon fixation in the light ($^{13}A_x$) (Fig. 6a, b). In *A. hypochondriacus*, rates of $^{13}CO_2^*$ entering the leaf were significantly different among treatments under high irradiance (>500 μmol quanta m^{-2} s^{-1}) (P<0.001) (Fig. 5a). In *B. napus*, under high irradiance (>800 μmol quanta m^{-2}

s^{-1}), there were significant differences among all [$^{13}CO_2^*$] (Fig. 5c). In *A. hypochondriacus* the highest rate of transpiration was ~3.5 mmol H_2O m^{-2} s^{-1} across all [$^{13}CO_2^*$]; however, in *B. napus* the highest rate of transpiration was ~4.5 mmol H_2O m^{-2} s^{-1} allowing more $^{13}CO_2^*$ to enter the cut leaves of *B. napus* compared to *A. hypochondriacus*. The amount of $^{13}CO_2^*$ entering the cut leaves was significantly different between the species in the MC and HC treatments across all irradiances above the light compensation point (P<0.001); however, the LC treatment was not significantly different between species across any irradiance.

In the CO_2-response curves, for both species, rates of $^{13}CO_2^*$ entering the leaf were highest when the rate of transpiration and stomatal conductance were highest (Fig. 5b, d). In *A. hypochondriacus*, the amount of $^{13}CO_2^*$ entering the leaf was significantly different in the LC treatment compared to the MC and HC treatments across all intercellular [CO_2] (P<0.05) (Fig. 5b). In *B. napus*, the amount of $^{13}CO_2^*$ entering the leaf in the LC treatment was significantly different from the MC and HC treatments across all [CO_2] (P<0.001). The amount of $^{13}CO_2^*$ entering the leaf was significantly different between species in the HC treatment across all [CO_2] (P<0.05) but there were no significant differences between species in the LC treatment across any [CO_2].

Rates of carbon fixation in the light

Rates of carbon fixation in the light increased with increasing irradiance and [$^{13}CO_2^*$] (Fig. 6a). Rates of carbon fixation were significantly different between the LC and HC treatments across all irradiances (P<0.001) and all treatments were significantly different when irradiance was greater than 1000 μmol quanta m^{-2} s^{-1} (P<0.001).

In the CO_2-response curves, rates of $^{13}A_{obs}$ increased with increasing intercellular [CO_2] in the KCl treatment (Fig. 6b); rates of $^{13}A_{obs}$ were ~1.1% the rate of $^{12}A_{obs}$. However, rates of carbon fixation in the light peaked at an intercellular [CO_2] of ~50 μmol mol^{-1} (Fig. 6b). Rates of carbon fixation in the light were significantly different across [CO_2] (P<0.05) and among [$^{13}CO_2^*$] (P<0.001).

The highest percent contribution of $^{13}CO_2^*$ to total assimilation was 6% which occurred under low irradiance and decreased with increasing irradiance and saturated at

~2.2% in *A. hypochondriacus* (Fig. S1). In *B. napus*, but the highest percentage of $^{13}CO_2^*$ was only 3% but under saturating irradiance was ~1.8%. However, the percent of $^{13}CO_2^*$ consumed by enzymes in the light was three times higher in *A. hypochondriacus* compared to *B. napus* across all irradiances (Fig. S2).

DISCUSSION

Fate of xylem-transported CO_2 in the dark

Our method, of providing a cut leaf of the Kranz-type C_4 species, *Amaranthus hypochondriacus*, a solution of [$^{13}CO_2^*$] (the sum of $[CO_2]_{aq}$, $[H_2CO_2]$, $[HCO_3^-]$ and $[CO_3^{2-}]$) and placing the leaf in a custom LI-6400 leaf chamber coupled to a tunable diode laser absorption spectroscope (TDL), allowed us to measure the rate of $^{13}CO_2$ efflux out of the leaf in the dark among all [$^{13}CO_2^*$]. As with C_3 species (Stutz *et al*., 2017), rates of $^{13}C_{efflux}$ in *A. hypochondriacus*, were dependent upon the rate of transpiration and the [$^{13}CO_2^*$], with the highest rates of $^{13}C_{efflux}$ occurring when both the rate of transpiration and [$^{13}CO_2^*$] were high (Fig. 1b). However, compared to the herbaceous C_3 species, *Brassica napus* (Stutz *et al*., 2017), rates of $^{13}C_{efflux}$ were ~2 times lower in *A. hypochondriacus* at the same rate of transpiration in the high-carbon (HC) treatment (Fig. 1b, c).

The highest percentage of xylem-transported CO_2 that exited the cut leaf of *A. hypochondriacus* as CO_2 was 30%, which occurred in the medium-carbon (MC) and HC treatments (Fig. 3a). However, this percentage is much lower than what is observed in C_3 species, 75% in *B. napus* (Fig. 3b) and 80% in *P. deltoides* (Stringer & Kimmerer, 1993; Stutz *et al*., 2017). Due to the low efflux of xylem-transported CO_2 from *A. hypochondriacus* leaves, the rate of xylem-transported CO_2 retention was higher in *A. hypochondriacus* (~0.25 μmol CO_2 m^{-2} s^{-1}) compared to *B. napus* (~0.08 μmol CO_2 m^{-2} s^{-1}), in the first 90 minutes after turning off the light, indicating one of several possibilities: 1) lower rates of transpiration, caused by lower stomatal conductance (g_s) in *A. hypochondriacus* allowed for more time for carboxylases to capture the inorganic carbon, 2) slow deactivation of a larger pool of PEPC in *A. hypochondriacus*, or 3) Kranz anatomy providing a physical barrier to xylem-transported CO_2 exiting the leaf.

Stomatal conductance before cutting the leaf was ~0.5 mol H_2O m^{-2} s^{-1} for both species. However, after turning off the light in *A. hypochondriacus* g_s declined to ~0.008 mol H_2O m^{-2} s^{-1} within an hour (data not shown) while g_s in *B. napus* declined to ~0.08 mol H_2O m^{-2} s^{-1}. Night g_s was higher in C_3 species (0.1 mmol H_2O m^{-2} s^{-1}) compared to Kranz-type C_4 *Atriplex* shrubs and a Kranz-type C_4 grass (~0.03 mmol H_2O m^{-2} s^{-1}) in warm deserts (Snyder *et al*., 2003). The dark g_s we observed in *A. hypochondriacus* was considerably lower than g_s on attached leaves from closely related C_4 species. It is likely that the low g_s observed in our experiment is the result of cutting the leaf. Recent work shows night g_s and rates of dark transpiration are higher than previously thought in both C_3 and C_4 species (Caird *et al*., 2007; Resco de Dios *et al*., 2015). Despite lower g_s, a higher vapor pressure difference (VPD) kept transpiration rates for *A. hypochondriacus* similar to those for *B. napus*. However, $^{13}C_{efflux}$ was lower in *A. hypochondriacus* compared to *B. napus* making it unlikely that lower stomatal conductance caused the difference in $^{13}C_{efflux}$, and increasing the likelihood that slower carboxylase deactivation in the dark might be responsible for the difference in $^{13}C_{efflux}$ between *A. hypochondriacus* and *B. napus*.

Once carboxylases are deactivated in the dark, 90 to 120 minutes in C_4 species and 30 to 60 minutes in C_3 species (Rajagopalan *et al.*, 1993), the consumption of $^{13}CO_2^*$ (the sum of $[CO_2]_{aq}$, $[H_2CO_3]$, $[HCO_3^-]$ and $[CO_3^{2-}]$) should be at a minimum. Consistent with that idea, we found that when the light has been off for at least 120 minutes, the consumption of $^{13}CO_2^*$ in the dark was similar between *B. napus* (~0.07 μmol CO_2 m^{-2} s^{-1}) and *A. hypochondriacus* (~0.07 μmol CO_2 m^{-2} s^{-1}); despite *in vitro* PEPC activities being more than 30 times higher in the light in C_4 species compared to C_3 species (Alonso-Cantabrana & von Caemmerer, 2016). However, there was no increase in $^{13}C_{efflux}$ between 30 and 120 min in the dark that one might expect after the carboxylases deactivate, but the potential signal was low since transpiration was low at that time.

The vascular bundles of Kranz-type C_4 plants are tightly surrounded by bundle-sheath cells which in turn are tightly surrounded by mesophyll cells. For $^{13}CO_2^*$ to exit the leaf of *A. hypochondriacus* it must pass through the layer of bundle-sheath followed by mesophyll cells (Fig. 7). However, in C_3 species, the leaf is differentiated into an upper palisade and loosely compact spongy mesophyll cells with a high surface area exposed to the intercellular airspaces (Busch *et al*., 2012; Giuliani *et al*., 2013), as well as non-

photosynthetic bundle-sheath cells surrounding the vascular bundle (Holaday *et al*., 1984). Therefore, Kranz anatomy likely provides a physical barrier that limits the efflux of xylem-transported CO_2 out of *A. hypochondriacus* in the dark. Consistent with that, we found that following 120 minutes of dark accumulation and under similar rates of transpiration the rate of $^{13}C_{efflux}$ is at least three times greater in *B. napus* compared to *A. hypochondriacs* (Fig. 1c) across the same transpiration.

Use of xylem-transported CO_2 in the light

Our method, of placing a cut Kranz-type C_4 leaf in a solution of [$^{13}CO_2^*$] allowed us to estimate how much $^{13}CO_2^*$ was used for carbon fixation in the light. Although we believe the $^{13}CO_2^*$ was captured by Rubisco and PEPC for photosynthesis, it could have been used for non-photosynthetic reactions (DiMario *et al*., 2018) and our methods did not distinguish between those fates. Photosynthetic discrimination ($\Delta^{13}C$) confirms that little if any xylem-transported CO_2 exited the leaf in the low-carbon (LC) treatment (Fig. 4a, b); however, some xylem-transported CO_2 exited the leaf in the MC and HC treatments as evidenced by the high $\Delta^{13}C$ values observed in both the light- and CO_2-response curves (Fig. 4c, d).

As expected, the rate of $^{13}CO_2^*$ entering the leaf was dependent upon the rate of transpiration and [$^{13}CO_2^*$] for both species (Fig. 5). Rates of transpiration were higher in *B. napus* compared to *A. hypochondriacus* which led to higher rates of $^{13}CO_2^*$ entering *B. napus* leaves (Fig. 5). However, we detected little xylem-transported CO_2 exiting the leaf of *A. hypochondriacus* in the light. Therefore, the capacity for *A. hypochondriacus* to use xylem-transported CO_2 is greater than the capacity for C_3 plants, either via photosynthesis or other enzymatic processes that consume CO_2 in the light (see Table 2 in DiMario *et al.*, 2018). Concentrations of CO_2^* are high in tree stems (Teskey *et al*., 2008); yet prior work shows little xylem-transported CO_2 is used for photosynthesis compared to what is available (Bloemen *et al*., 2013a). However, this was not the case for Kranz-type C_4 plants, especially at lower $^{13}CO_2^*$ concentrations.

Across all treatments, in the light, little xylem-transported CO_2 exited the leaves, indicating that nearly all $^{13}CO_2^*$ was consumed. $^{13}CO_2^*$ exiting the vascular bundles of Kranz-

type C_4 leaves may exit the leaf by (Fig. 7): 1) traveling apoplastically, along the cells walls of bundle-sheath (BSC) and/or mesophyll cells (MC), 2) traveling symplastically, through the interior of BSCs and/or MCs, or 3) traveling in gas phase (Buckley, 2015). The amount of diffusion through each of these pathways is unclear, owing in part to a lack of knowledge about aquaporins and the location of carbonic anhydrases (CA). Aquaporins allow water to quickly and easily pass through membranes but also facilitate the passage of CO_2 and other small uncharged molecules (Uehlein *et al*., 2003; Flexas *et al*., 2006; Maurel *et al*., 2008; Sade *et al*., 2013), while CAs will facilitate equilibrium between pools of inorganic carbon. CA has not been firmly localized to the apoplast in higher plants, though it has been isolated in the cell walls of *Chlamydomonas reinhardtii* (Moroney *et al*., 2011), and is known to associate with the plasma membrane (DiMario *et al*. 2017). If apoplastic CAs are discovered in the future, it will suggest that significant apoplastic transport of CO_2^* is possible, otherwise, it is likely that most transport is via symplastic routes. In C_4 plants CA activities are ~4 times higher in MCs than BSCs (DiMario *et al*., 2017). $^{13}CO_2^*$ that diffuses past the BSCs to the MCs may be kept at equilibrium by CA, maintaining the supply of CO_2 for Rubisco. $^{13}CO_2^*$ that diffuses past the BSCs to the MCs could further be fixed by PEPC and returned to the BSC as a C_4 acid (Fig. 7). Dissolved xylem-transported CO_2^* traveling with water symplastically through the cells would be captured by photosynthetic cells but not be detected with our TDL technique. Alternatively, if dissolved $^{13}CO_2^*$ travels apoplastically through the leaf or via the gas phase, it will enter the intercellular air space, where it can be captured by PEPC or exit the leaf as CO_2. Our method with the TDL does not allow us to differentiate possible paths for $^{13}CO_2^*$ traveling through a leaf or if photosynthetic enzymes or other enzymes consumed the $^{13}CO_2^*$. However, with our method we know how much $^{13}CO_2^*$ enters a leaf and how much $^{13}CO_2$ exits a leaf.

Significance for the evolution to C_4 photosynthesis?

C_4 photosynthesis is spread across 19 plants families reflecting 61 independent origins (Sage, 2016) in both eudicots and monocots. C_4 plants have a separation of C_3 and C_4 enzymes, which occurs in one of two ways: 1) Kranz-type C_4 plants that separate Rubisco and PEPC into two distinct cells, bundle-sheath and mesophyll respectively (Edwards *et al*.,

2004) or 2) single-cell C_4 plants that use a single-cell with partitions for C_3 and C_4 enzymes (Voznesenskaya *et al*., 2001; Edwards *et al*., 2004). Compared to C_3 species, intermediate C_3-C_4 and Kranz-type C_4 plants have increased vein densities, reduction in intercellular airspaces, and enhancement of bundle-sheath organelles (Monson, 1999; Sage, 2004). While increasing vein densities may only increase structural integrity or enhance leaf water status (Sage, 2004) it may also allow xylem-transported CO_2 to be utilized for photosynthesis by reducing the distance between vascular bundles transporting xylem-transported CO_2 and photosynthetic cells.

Kranz syndrome is characterized by Kranz cells with numerous chloroplasts, thick outer walls, tight packing around the vascular bundles and limited exposure to intercellular air spaces (Sage *et al*., 2014). Tight packing of cells around the vascular bundles will limit the amount of xylem-transported CO_2 that diffuses into the intercellular airspace and eventually out of the leaf.

Additionally, tightly packed cells around the vascular bundle will provide increased hydraulic continuity and cavitation repair in the leaf, along with other benefits (see Griffiths *et al*., 2013). Thus a well-developed system for capturing xylem-transported CO_2 would also provide the hydraulic benefits which allow Kranz-type C_4 plants to survive in environments with higher evaporative demand without suffering the consequences of hydraulic failure (Osborne & Sack, 2012). Furthermore, the stems of herbaceous C_3 plants have been hypothesized to exhibit an analogous process to Kranz anatomy where malate, dissolved in xylem sap, is taken up by the CO_2 limited cells surrounding the vasculature in the stems and then delivered as malate to photosynthetic cells (Hibberd & Quick, 2002). Higher evaporative demand in the leaf will increase the vasculature of the leaf to decrease embolisms but will also allow cells surrounding the vascular bundles access to carbon dissolved in xylem water in a process similar to the transfer of malate from MC to BSC in C_4 plants. The coupling of increased veins to reduce embolism along with the supply of inorganic carbon from vascular bundles may have allowed for the multiple independent origins of C_4 photosynthesis proposed by Hibberd & Quick (2002).

We observed little xylem-transported CO_2 exiting the leaves of the Kranz-type C_4 species compared to C_3 species indicating that Kranz-type C_4 plants have a higher capacity to

consume xylem-transported CO_2 compared to C_3 species. Kranz anatomy is likely the cause for the difference in $^{13}C_{efflux}$ in the dark and $^{13}C_{light\ efflux}$ in the light between C_3 and C_4 plants. We believe that Kranz anatomy effectively limits xylem-transported CO_2 from exiting leaves and thus allowed *A. hypochondriacus* to use nearly 75% of the $^{13}CO_2^*$ compared to *B. napus* which used only 30% of the $^{13}CO_2^*$ (Fig. S2). However, due to differences in total assimilation the contribution of $^{13}CO_2^*$ to total assimilation was similar between the two species (Fig. S1). Thus, the ability of Kranz anatomy to capture xylem-transported CO_2 provides Kranz-type C_4 plants another way to increase their carbon use efficiency over their C_3 counterparts.

CONCLUSION

Our technique, using a LI-6400 coupled to a TDL, allowed us to measure the efflux of xylem-transported CO_2 out of cut leaves in the Kranz-type C_4 species, *Amaranthus hypochondriacus* in the dark. Rates of $^{13}C_{efflux}$ in the dark increased with increasing rates of transpiration and [$^{13}CO_2^*$]. However, when compared to a herbaceous C_3 species, *Brassica napus,* at the same rate of transpiration and [$^{13}CO_2^*$], rates of $^{13}C_{efflux}$ were lower in *A. hypochondriacus* indicating that leaf anatomy or enzymatic activity in the dark decreased the flux of xylem-transported CO_2 out of the leaf. In the light, the amount of xylem-transported CO_2 entering the leaf was dependent upon the rate of transpiration and [$^{13}CO_2^*$]. We were not able to detect any xylem-transported CO_2 exiting the leaf in the LC treatment using photosynthetic discrimination, but in the MC and HC treatments, photosynthetic discrimination showed that xylem-transported CO_2 exited the leaf. However, the low efflux rate of xylem-transported CO_2 in the light indicates that *A. hypochondriacus* is likely able to recapture and use nearly all xylem-transported CO_2 for photosynthesis, achieving rates of carbon capture that are up to 6% of photosynthesis, similar to C_3 plants at equivalent transpiration rates. However, *A. hypochondriacus* was able to use up to 75% of xylem-transported CO_2 supplied while *B. napus* only used up to 25% of the xylem-transported CO_2. Compared to the C_3 species, the amount of xylem-transported CO_2 exiting the cut leaves of *A. hypochondriacus* was small, indicating that Kranz-type C_4 species are able to utilize more xylem-transported CO_2 than their C_3 counterparts. Kranz

anatomy is another way C_4 plants are able to increase their carbon use efficiency over C_3 plants.

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AUTHOR CONTRIBUTIONS

S.S.S. performed experiments and analyzed data. D.T.H. provided a conceptual framework. S.S.S. and D.T.H. wrote the manuscript.

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Supporting information (SI)

Table S1 Mean pH and standard deviation (±1 SD) for the dark, light- and CO_2-responses in *Amaranthus hypochondriacus*.

Table S2 Mean pH and standard deviation (±1 SD) for the dark, light- and CO_2-responses in *Brassica napus*.

Fig. S1 The contribution of xylem-transported CO_2 assimilation to total assimilation across a light-response curve from the HC treatment for *A. hypochondriacus* and *B. napus*.

Fig. S2 The percent of $^{13}CO_2^*$ consumed by enzymes in the light across a light-response curve from the HC treatment for *A. hypochondriacus* and *B. napus*.

FIGURES

Fig. 1 (a) Transpiration dependence of leaf respiration ($^{12}C_{RL}$) for three [$^{13}CO_2^*$]—light gray squares represents the low-carbon (LC) treatment (y=0.1413x + 1.234, R^2=0.1337), dark gray squares represent the medium-carbon (MC) treatment (y=0.2046x + 1.215, R^2=0.3945) and filled squares represent the high-carbon (HC) treatment (y=0.3013x + 1.048, R^2=0.7326). (b) Transpiration dependence of $^{13}C_{efflux}$ in the dark for three [$^{13}CO_2^*$] light gray circles represent the LC (y=0.002446x + 0.01554, R^2=0.2740), dark gray circles represent the MC (y=0.04432x + 0.001913, R^2=0.9019) and filled circles represent the HC (y=0.06925x -0.007001, R^2=0.9661). (c) Transpiration dependence of $^{13}C_{efflux}$ in the HC treatment from *B. napus* (dark green circles) and *A. hypochondriacus* (black circles) from the HC treatment. Measurements were made on excised leaves of *A. hypochondriacus* or *B. napus* placed in one of the three [$^{13}CO_2^*$] solutions. The low-carbon (LC) was 1.19 mmol l^{-1}, medium-carbon (MC) was 5.95 mmol l^{-1} and high-carbon (HC) was 11.9 mmol l^{-1}. Measurements represent averages and ±1 standard deviation (SD) of five replicates for *A. hypochondriacus* and four replicates for *B. napus*. Transpiration was averaged over 0.1 mmol H_2O m^{-2} s^{-1} increments and represent rates of transpiration and $^{13}C_{efflux}$ at least 45 minutes after the light was turned off. [$^{13}CO_2^*$] is the sum of the concentrations of all species of dissolved inorganic carbon in the xylem ($[CO_2]_{aq}$, $[H_2CO_2]$, $[HCO_3^-]$ and $[CO_3^{2-}]$). *B. napus* data modified from Stutz *et al*. (2017).

Fig. 2 Response of gross (a) efflux of xylem-transported CO_2, $^{13}C_{efflux}$ (circles) and (b) leaf respiration, $^{12}C_{RL}$ (squares) in *A. hypochondriacus* at a transpiration rate of 0.75 mmol H_2O m^{-2} s^{-1} for each [$^{13}CO_2^*$]. Measurements were made on cut leaves of *A. hypochondriacus* placed in one of three [$^{13}CO_2^*$] solutions—LC, MC, and HC. Measurements represent averages and ±1 standard deviation (SD) of five replicates for each [$^{13}CO_2^*$]. The low-carbon (LC) was 1.19 mmol l^{-1}, medium-carbon (MC) was 5.95 mmol l^{-1} and high-carbon (HC) was 11.9 mmol l^{-1}. [$^{13}CO_2^*$] is the sum of the concentrations of all species of dissolved inorganic carbon in the xylem ($[CO_2]_{aq}$, $[H_2CO_2]$, $[HCO_3^-]$ and $[CO_3^{2-}]$).

Fig. 3 The percentage of gross xylem-transported $^{13}CO_2$ exiting the leaf in the transpiration stream in (a) *A. hypochondriacus* and (b) *Brassica napus* across three [$^{13}CO_2^*$] averaged over the first 90 minutes after turning off the light. Estimated rates xylem-transported $^{13}CO_2$ consumption in cut leaves of (c) *A. hypochondriacus* and (d) *B. napus* across three [$^{13}CO_2^*$] averaged over the first 90 minutes after turning off the light. Measurements represent averages and ±1 standard deviation (SD) of five replicates for *A. hypochondriacus* and four replicates for *B. napus* made on cut leaves under the following concentrations: LC, MC, and HC. The low-carbon (LC) was 1.19 mmol l^{-1}, medium-carbon (MC) was 5.95 mmol l^{-1} and high-carbon (HC) was 11.9 mmol l^{-1}. *B. napus* data modified from Stutz *et al*. (2017). [$^{13}CO_2^*$] is the sum of the concentrations of all species of dissolved inorganic carbon in the xylem ([CO_2]$_{aq}$, [H_2CO_2], [HCO_3^-] and [CO_3^{2-}]).

Fig. 4 Photosynthetic discrimination ($\Delta^{13}C$) across (a,c) light- and (b, d) CO_2-response curves in *Amaranthus hypochondriacus*. Light-response curves (a) KCl control (open diamonds) and LC (light gray diamonds) and (c) MC (dark gray diamonds) and HC (filled diamonds) treatments. CO_2-response curves (b) KCl control (open diamonds) and LC (light gray diamonds) and (d) MC (dark gray diamonds) and HC (filled diamonds) treatments. Measurements represent means and ±1 standard deviation (SD) of five replicates for each treatment on cut leaves of *A. hypochondriacus*. The low-carbon (LC) was 1.19 mmol l^{-1}, medium-carbon (MC) was 5.95 mmol l^{-1} and high-carbon (HC) was 11.9 mmol l^{-1}.

Fig. 5 Calculated rates of $^{13}CO_2^*$ entering cut leaves of (a, b) the C_4 species *A. hypochondriacus* and (c, d) the C_3 species *B. napus* in (a, c) light- and (b, d) CO_2-response curves. LC (light gray triangles), MC (dark gray triangles) and HC (closed triangles) for both species. Measurements represent means and ±1 standard deviation (SD) of five replicates for each treatment in *A. hypochondriacus* and *B. napus*. The low-carbon (LC) was 1.19 mmol l^{-1}, medium-carbon (MC) was 5.95 mmol l^{-1} and high-carbon (HC) was 11.9 mmol l^{-1}. $^{13}CO_2^*$ is the sum of all species of dissolved inorganic carbon in the xylem ([CO_2]$_{aq}$, [H_2CO_2], [HCO_3^-], and [CO_3^{2-}]).

Fig. 6 Light- and CO_2-response curves for rates of photosynthesis with (a, b) ^{13}Carbon ($^{13}A_x$—photosynthesis using xylem-transported CO_2, or background rates of $^{13}A_{obs}$ in KCl treatment) and (c, d) ^{12}Carbon ($^{12}A_{obs}$—photosynthesis using CO_2 derived from the atmosphere). $^{13}A_x$ in *A. hypochondriacus* KCl (open circles), LC (light gray circles), MC (dark gray circles) and HC (closed circles) and $^{12}A_{obs}$ for *A. hypochondriacus*, for KCl (open squares), LC (light gray squares), MC (dark gray squares) and HC (closed squares). Measurements represent means and ±1 standard deviation (SD) of five replicates for each treatment. The low-carbon (LC) was 1.19 mmol l^{-1}, medium-carbon (MC) was 5.95 mmol l^{-1} and high-carbon (HC) was 11.9 mmol l^{-1}.

Fig. 7. The fate of xylem-transported CO_2 in Kranz-type C_4 plants. Inorganic carbon ($[CO_2^*]$, the sum of $[CO_2]_{aq}$, $[H_2CO_3]$, $[HCO_3^-]$ and $[CO_3^{2-}]$) dissolved in xylem water (blue on the figure) travels from the roots to the leaves of the plant likely interacting with carbonic anhydrase in multiple locations in leaves, keeping each species in equilibrium. In the dark, xylem-transported CO_2 can move apoplastically along the cell walls of the bundle-sheath and mesophyll cells before entering the substomatal cavity and diffusing out of the leaf as CO_2 (red arrow). The transpiration stream may also move xylem-transported CO_2 symplastically through the bundle-sheath and mesophyll cells and be consumed by non-photosynthetic processes occurring in the dark (pink arrows). In the light, xylem-transported CO_2 can also enter cells bundle sheath cells and be captured by Rubisco for photosynthesis, or enter the mesophyll cells and be captured by PEPC before being transported to Rubisco for photosynthesis. As in the dark, xylem-transported CO_2 can also continue through the apoplast to the substomatal cavity and diffuse out of the leaf.